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13. ABSTRACT *(Maximum 200 Words)*

Our main goal in this study is to evaluate the sensitivity of primary breast cancer cells (PBCCs) grown both in vitro and *in vivo* (nude mice) to retinoids. Contrary to most previous studies on established breast cancer cell lines, in this study we assessed the effects of retinoids (*all-trans* retinoic acid (atRA), *9-cis* retinoic acid (9cisRA) and 4-(hydroxyphenyl)retinamide (4-HPR) on PBCCs grown both, *in vitro* (first several passages) and in nude mice. Tissue samples from 16 breast carcinomas were cultured *in vitro* and in 10 of them (62.5%) satisfactory growth was found. We optimized the protocol for assessment ER, retinoic acid receptors (RAR-α, β, γ), and retinoid X receptors (RXR-α, β, γ) in paraffin sections from breast tumors as well as in cells growing *in vitro*. We found that 7 from 16 tumors were ER negative, that RARα was expressed in 14, RARβ in 10, RARγ in 15, from 16 tumors respectively. In 60% of tumors RARβ was expressed both in nucleus and cytoplasm. RXRαa and RXRγ were also expressed both in 15 from 16 tumors. Cells from 5 tumors were implanted in nude mice and 4 indicated satisfactory growth. Tumor cells cultured in vitro, as well as growing in nude mice are currently under investigation for assessment the effects of various retinoids on cell proliferation and cell death-related markers.

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Table of Contents:	**Page#**

Sensitivity of Breast Cancer to Retinoids. Potential Clinical Implications

Introduction:

Our main goal in this study is to evaluate the sensitivity of primary breast cancer cells (PBCCs) to retinoids. The rational for estimating the sensitivity of tumor cells to various retinoids is motivated by the increasing evidences that retinoids can not only inhibit mammary carcinogenesis in animal models but can also suppress the occurrence of breast cancer in women with increased risk of developing the disease (1). Recent results from a breast cancer chemoprevention clinical trial in Italy showed that 4-(hydroxyphenyl) retinamide (4-HPR), a synthetic analog of Vitamin A, when given for 5-8 years to women with removed primary breast cancer, reduced the occurrence of cancer in contralateral breast with about 30% in premenopausal women only. The same retinoid did not work in postmenopausal women, suggesting that hormone environment may substantially modulate the chemopreventive efficacy of retinoids. It is still not known whether 4-HPR or other Vitamin A analogs may have beneficial effect in women with already developed breast cancer. Preclinical and clinical studies are in progress with other retinoids: 9-cis retinoic acid (9cRA) and Targretin that may also have inhibitory effect on breast cancer development (2,3).

The effects of retinoids are mainly mediated by two classes of nuclear retinoid receptors that are members of the steroid hormone receptor super family that also includes estrogen, thyroid hormone, and Vitamin D receptors (4,5). The nuclear retinoid receptors are divided into retinoic acid receptors (RARs) and retinoid X receptors (RXR) that are both composed of subtypes; α, β, and γ. Each of the subtypes exhibits specific pattern of expression in various tissues during embryonic development as well as during carcinogenesis. Defects in retinoid receptor structure, expression, and function have been detected in various types of cancer cells. It has been suggested that they may enhance cancer development by interfering with retinoid signaling pathways, thereby abrogating the putative physiological anticarcinogenic effects of natural retinoids (6,7). Loss of RARβ has been reported to occur in premalignant and malignant stages of different forms of cancer, including breast cancer. Restoration of RARβ function may suppress or reverse the neoplastic process (5).

In this project we will characterized RAR α, β, γ and RXR α, β, γ expression in breast tumor samples as well as in PBCCs grown *in vitro* as well as in nude mice. We will introduce an *in vitro/in vivo* approach to assess the sensitivity of PBCCs to atRA, which affects RAR-α, to 9cRA, which is ligand for both RARs and RXRs, and to 4-HPR, which seems to work without affecting neither RARs nor RXRs. Contrary to most previous studies on established breast cancer cell lines, we will focused on PBCCs, because they are heterogeneous and their phenotype appears to be closer to the primary tumor than to already established tumor cell lines.

For *in vivo* experiments PBCCs grown *in vitro* (first several passages) will be mixed with Matrigel, and transplanted in mammary gland parenchyma of nude mice. When tumor outgrowths occur, the animals will be treated for four weeks with 4-HPR or 9cRA. The response of palpable tumors to retinoids will be assessed by changes in tumor volume,

expression of cell differentiation markers (cytokeratins), in the percent of proliferating and apoptotic cells. By these *in vitro/in vivo* approach we will identify tumors sensitive or resistant to specific retinoid (s). The most sensitive tumors to retinoids should be those with the highest percentage of decrease in the total number of cells and in the proliferating cells and/or with the highest index of apoptotic cells. This information may help in developing of new strategy for selection of patients and retinoids for chemoprevention or therapy trials.

Technical Objectives

We will

1. Develop an *in vitro* assay for assessment the effects of retinoids on primary breast cancer cells PBCCs
2. Assess whether RARs and RXRs status of breast tumor samples and PBCCs may affect their response to atRA, 9cRA, and 4-HPR
3. Estimate whether there is relevance in the response of PBCCs to the above retinoids when cultured *in vitro* and when transplanted in nude mice

Statement of work

Task 1: Months 1-3: Updating of the specific aims, planning of the experiments, analysis of the individual projects, preliminary experiments for optimizing the *in vitro* work and the experiments with collagen gel

Task 2: Months 4-8: Initiation of work with breast tumors. Optimize the procedures for identification of RARs, RXRs, ER, BrdU, and apoptosis. Initiation of the work with nude animals

Task 3: Months 9-12: Evaluation of the effect of retinoids on the first 10-15 PBCCs grown *in vitro*.

Results

a. Growth of primary breast cancer cells (PBCCs) *in vitro*

We optimize the protocol for culturing of PBCCs *in vitro* (Appendix I, Protocol 1). In addition to collagen gel system, tumor fragments and trypsinized tumor cells were cultured. Because of the small tumor samples obtained from the surgical room, in most cases it was difficult to generate sufficient number of cells for collage gel assay. For this reason, small tumor fragments were also used to initiate the cell growth *in vitro*. Cells were grown in tissue culture plates and when became confluent they were co-cultured in 24 well plates for assessment the cell number, their morphology, ER, RARs and RXRs status. In most cases between the 3-8 passage were used to perform various assays. As shown in Table 1(Appendix 1) in 10 from 16 tumors (62.5%) more than 4 passages were achieved and sufficient number of cells generated for *in vitro* and *in vivo* assays.

In the preliminary data that we have with five PBCCs growing *in vitro* and treated with atRA, 9cRA, or 4-HPR in 3 of them 9cRA was most efficacious in inhibiting cell

growth and in inducing cytopathological alterations: detachment from the growing surface, increase in cell size, occurrence of gigantic cells or multinuclear cells, as well as occurrence of cells with pyknotic and apoptotic nuclei. From the several concentrations tested: 10^{-7} M, 10^{-6} M, 10^{-5} M, we selected 5 x 10^{-6} M as most efficacious in suppressing cell growth, but not inducing massive cell death. Both control and treated with retinoids cells grew in a parallel way within the first 3 days. Between the 3rd and 6th day in most cells treated with retinoids a decrease in cell number in the range of 30-50% was found.

b. RARs and RXRs status in breast tumor samples

In all tumors cultured in vitro ER, RAR α, β, γ, and RXR α, β, γ were evaluated by ICH and in some cases by Western blot. Summarize data on the expression of various receptors are given in Table 1 and Table 2. Northern blot and/or RT-PCR procedures for assessment the functional status of the above receptors and their modulations by retinoids are in a process of optimization. From 16 tumors examined 7 were ER negative and the remaining were ER positive. The number of ER positive nuclei and the intensity of staining were quite variable in individual tumors. RARα was expressed in 14 from 16 breast tumors. The protein was entirely localized in the nucleus (Appendix II, Fig. 1A). Most of tumor cells were positively stained for RARα. RARβ was found in 10 tumors. Six tumors were negative and this was confirmed by Western blot. In 6 from 10 tumors RARβ was expressed in the nucleus and cytoplasm or entirely in the cytoplasm (Appendix II, Fig. 1b and c). Surprisingly, a high level of RARβ expression was found in myoepithelial cells (Fig. 1B, arrow). RARγ was identified in 15 from 16 tumors examined. RARγ was localized in the nucleus or both, in the nucleus and cytoplasm of tumor cells ((Appendix II, Fig. D and E). The level of expression was variable between individual cells. Among the RXR most consistent results were obtained for RXRα and RXRγ (Table 1 and 2). RXRα was identified in 15 tumors; one tumor only was negative for RXRα. In most tumors, RXRα on was also observed (Appendix I, Table 2 and Appendix II, Fig. F).
We still have problems in identifying RXRβ in most tumors. RXRβ antibody from Santa Cruz Biotech. Co., Santa Cruz, CA. worked in some tumors, but did not in all of them. As shown in Table 1 and 2 from 11 tumors examined, 5 tumors were negative, in 4 tumors the protein was localized in the cytoplasm and in 1 tumor, in the nucleus, (Appendix II, Fig. G). RXRγ was expressed in most tumors (14 from 15 examined). The protein was observed mostly in the nucleus, but there were tumors with both, nuclear and cytoplasmic expression of the receptor (Appendix II, Fig. H).

Tumor cells grown in vitro preserved their ER, RARs and RXRs status. In addition to the cells that expressed high level of the proteins in the nucleus, there were cells in which both, nuclear and cytoplasmic staining as well as preferential cytoplasmic staining was observed (Appendix II, Fig. 2A-F).

c. Effects of retinoids in nude mouse implanted PBCCs

From 4 tumors we generated sufficient number of cells (5 x 10^5) to mix with Matrigel and implant into mammary gland parenchyma of nude mice. Abdominal and thoracic mammary glands were used. Matrigel was mixed 1:1 with cell suspension. Three tumors indicated growth in nude mice, starting 2-3 weeks after implantation and were used for treatment with retinoids. The animals were divided in control and treated groups

(at lest 4 animals per group with 10 tumors). 4-HPR and 9cRA only were used for *in vivo* experiments. 4-HPR was given at784 mg/kg and 9cRA at 100 mg/kg diet. Control animals were put on a placebo diet. Retinoids are given for 4 weeks starting after palpable tumors occur. Before sacrifice, the animals will be injected with BrdU, 50 mg/kg b.w. for labeling of proliferating cells. Tumor growth is monitored weekly by measuring the large (**a**) and small (**b**) diameters. Changes in tumor volume (**V**) will be calculated by the formula: $\mathbf{V} = \mathbf{a} \times \mathbf{b}^2/2$. Two hours prior sacrifice the animals will be injected i.p.with BrdU (50 mg/kg b.w.) for labeling of proliferating cells. These experiments are still in progress and we do not have the final results.

Key Research Accomplishments

- We found that in about 60% of breast carcinomas PBCCs could be successfully cultured for several passages *in vitro*. The cells preserved their heterogeneity, including the expression of RAR α, β, γ, and RXR α, β, γ.
- We characterized ER, RAR α, β, γ, and RXR α, β, γ status in breast tumor samples fixed in formalin and embedded in paraffin (Appendix II, Fig. 1) as well as in primary breast cancer cells grown *in vitro* (Appendix II, Fig. 2).
- RARβ was not expressed in about 40% of tumors, whereas the other receptors (with exception of RXRb) were identified in most tumors. In the remaining tumors both, nuclear and cytoplasmic localization of RARβ was observed.
- We found that atRA, 9cRA and 4-HPR suppress cell growth *in vitro*, starting 3 days after initiation of treatment. Doses in the range of 5×10^{-6} to 2×10^{-6} M appear to be most appropriate for inhibition the growth of PBCCs *in vitro*.
- In vitro growing PBCCs when mixed with Matrigel could be successfully implanted in nude mice. Tumors that develop in nude mice could be used for various therapy related studies.

Reportable Outcome

At this time point we do not have sufficient data to be published.

Conclusions

We developed an *in vitro/in vivo* assay for assessment of sensitivity of primary breast cancer cells (PBCCs) to atRA, 9cRA and 4-HPR. More tumors need to be examined in order a correlation between RARs and RXRs status of tumor cells and their sensitivity to retinoids to be assessed.

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Appendix I

Protocols: 1-5

Tables: 1-2

Protocol #1:

Outlines for breast cancer tissue examination

***In vitro* studies:**

1. Obtain fresh tumor tissue from the surgical room
2. Remove the fat and necrotic areas (if you need help, please contact Dr. K. Christov)
3. Cut small peace from tumor (100-300 mg, 2-3 mm) and put it in liquid nitrogen. This tissue will be used later for flow cytometry (FCM) and for estimation of RARs and RXRs.
4. Cut the tissue in many (as many as possible) tissue aggregates and allow them growing in vitro for several days. If possible use the first generation for assessment the efficacy of chemopreventive drugs, if not culture the cells for 2-4 generations and when achieve sufficient growth use the retinoids
5. Use cover sleeps for in vitro assay. Culture the cells in petri dishes. In each petri dish put 6 cover slips
6. Culture the cells for 5-7 days. Protect from light
7. Before termination of the experiment label the cells with BrdU (20 ug/ml) for 30 min. BrdU stock solution is sterile and kept at 4°C. Dissolve 10 mg BrdU (Sigma) in 5ml MEM wormed up to 37°C. Use 20 ul from the stock solution per 1 ml cell culture medium
8. At the end take the cover slips and fixe in 3% formaldehyde for 10 min followed by cold methanol and ethanol (5 min each one). For immunocytochemistry the cover sleeps could be fixed in aceton for 10 min, ethanol, 95%, and ethanol 70% and left in frizer. The oversleeps will be used for immunocytochemistry
9. Trypsinize the cells from the plate or petri dish, spin at 1000 for 5 min, wash in cold HEPES or PBS and fix in citrate buffer for flow cytometry

Effects of retinoids on cell growth

Groups/Treatment	Days of treatment*	
	3	7
O	3	3
RA	3	3
9-cis RA	3	3
4-HPR	3	3
	12	12

- three wells for each time point
- Evaluate the morphology of cells and their density
- Remove the cells by trypsin (0.05%) 5 min
- Count the cell number by cytometer Table 2

Effects of retinoids on cell proliferation and apoptosis**

Treatment Groups	Cover sleeps	BrdU-LI	Parameters Apoptosis	RARs/RXRs
0	6	+	+	+
RA	6	+	+	+
9-cisRA	6	+	+	+
4-HPR	6	+	+	+

** Petri dishes will be used
Used 4 petri dishes. Put 6-7 cover sleeps in each petri dish
Treat the cells for 7 days with retinoids
Labeled the cells for 1 hr with BrdU before fixing (50 ug/ml)
Remove the cover sleeps from the petri dish and fix them in aceton or formalin (4% neutral)

Retinoids:

A. Prepare a stock solution from RA, 4-HPR and 9-cis RA and keep it at –20°C. Protect from light at the time of preparation and during the experiment. Before use bring the stock solution at room temperature.
   - All trans retinoic acid (RA): 3 mg/ml ethanol (100% pure, Sigma) 0.01M
   - 4-HPR: 3.92 mg/ml ethanol (pure) – 0.01 M
   - 9-cis Retinoic acid: 3 mg/ml ethanol (pore) – 0.01 M

B. Prepare working solution
   - Take 20 ul from the stock solution and dissolve in 1 ml MEM (vortex)
   - Take 200 ul from the MEM and put in 20 ml medium for culturing
   - The final concentration is 2-5 x 10^{-6} M

Protocol : Effects of Retinoids on the growth of breast tumor cells in nude mice
In vivo studies:

- Trypsinize the cells
- Centrifuge at 1000g/5 min
- Wash in HEPES
- Count the cells in cell counter
- Mix 10^{-6} cells in 0.1 ml Matrigel
- Inject 0.1 ml Matrigel in 4 nude mice in 4 places: 2 abdominal and 2 thoracic mammary glands
- Weight until tumor growth occurs
- Start treating the animals with palpable tumors (2-3 mm diameter) with 4-HPR, 9-cisRA, or RA given in the diet for 4 weeks
- Measure tumor size once a week by caliper (a, b, c diameters). Do not press tumors when measure. You may induce mechanical damage of tumor parenchyma
- Before sacrifice inject the animals ip. with BrdU, 50 mg/kg body weight. No more than 0.1 ml per mouse

Take the tumor nodules out, cut them in two halves: one half fixe in 10% neutral formalin and the other freeze

PROTOCOL 2:

Assessment of the ER Status in Breast Carcinomas by Immunocytochemistry

Antibodies: ER Ab-14 (monoclonal) Neomarkers
Kit: Vecta Elite ABC Kit (mouse)
Slides from Formalin-fixed, Paraffin Blocks

Prepare 1% BSA in PBS (0.05g BSA + 5 ml PBS)
Deparaffinize Slides: 3 x 5 min Xylenes
2 x 3 min 100% Ethanol
2 min 95% Ethanol
2 min 80% Ethanol
2 x 3 min dH_2O
5 min 3% H_2O_2
2 x 3 min H_2O
Antigen Retrieval: Microwave slides in 250 ml citrate buffer 3 min (until Boiling) at 100% powers followed by 12 min at defrost (245 watts). Cool slides in citrate buffer 15 min. Rinse slides 2 x 3 min dH_2O.
Blot Slides dry and circle tissue with PAP Pen (Let circles dry 2 min)
5 min PBS
Blocking: 20 min normal blocking sol. ABC kit (3 drops stock sol.[goat] to 10 ml PBS) *(yellow bottle)*
Staining:
Incubate slides with Primary Ab 1 hour at room temp.
Antibody dilution: ER 1: 40 (30 μl Ab/ 1.2 ml PBS-BSA sol)
neg. control: nonimmune mouse IgG

3 x 5 min PBS (Make horse-anti-mouse and ABC at this time)
20 min Biotinylated Horse-antimouse IgG (10 ml PBS + 1drop Horse-antimouse, *blue bottle Vector Kit) Make sure real drop and not bubble!*
3x5 min PBS
30 min ABC (Vector Kit-10 ml PBS+2 drops A+2 drops B)
3x5 min PBS (Prepare DAB... 10 mg DAB/20 ml PBS, shake, filter, add 60 μl 3% H_2O_2)
2 min DAB
5 min dH_2O
Counterstain:
30 sec Gill's #1 hematoxylin
2 x 30 sec H_2O
20 sec Scott's tap H_2O substitute
5 min dH_2O
Mount:
1 min 70% EtOH, 1 min 95% EtOH, 2x1 min 100% EtOH
2x2 min Xylenes. Coverslip with Permount

PROTOCOL 3:

Assessment of the Expression of RARα, RARβ, RARγ in Breast Carcinomas by Immunocytochemistry

Antibodies: RARα, RARβ, RARγ (Santa Cruz) Polyclonal
Kit: Vecta Elite ABC Kit (rabbit)
Slides from Formalin-fixed Paraffin Blocks

Prepare 1% BSA in PBS (0.05g BSA + 5 ml PBS)
Deparaffinize Slides: 3 x 5 min Xylenes
2 x 3 min 100% Ethanol
2 min 95% Ethanol
2 min 80% Ethanol
2 x 3 min dH_2O
10 min 3% H_2O_2
2 x 3 min H_2O
Antigen Retrieval: Microwave slides in 250 ml citrate buffer 3 min (until Boiling) at 100% powers followed by 12 min at defrost (245 watts). Cool slides in citrate buffer 15 min. Rinse slides 2 x 3 min dH_2O.
Blot Slides dry and circle tissue with PAP Pen (Let circles dry 2 min)
5 min PBS
Blocking: 20 min normal blocking sol. ABC kit (3 drops stock sol.[goat] to 10 ml PBS) *(yellow bottle)*
Staining:
Incubate slides with Primary Ab 1 hour at room temp.
Antibody dilutions: RARα: 1:100 in 1% BSA in PBS
RARβ: 1:50 in 1% BSA in PBS
RARγ: 1:200 in 1% BSA in PBS
neg. control: nonimmune rabbit IgG
2 x 5 min PBS (Make goat-anti-rabbit and ABC at this time)
20 min Biotinylated Goat-antirabbit IgG (10 ml PBS + 25μl Goat-antirabbit, *blue bottle Vector Kit*) *Make sure real drop and not bubble!*
3x5 min PBS
30 min ABC (Vector Kit-10 ml PBS+2 drops A+2 drops B)
3x5 min PBS (Prepare DAB...10 mg DAB/20 ml PBS, shake, filter, add 60 μl 3% H_2O_2)
2 min DAB
5 min dH_2O
Counterstain:
10 sec Gill's #1 hematoxylin
2 x 30 sec H_2O
20 sec Scott's tap H_2O substitute
5 min dH_2O
Mount:
1 min 70% EtOH, 1 min 95% EtOH, 2x1 min 100% EtOH
2x2 min Xylenes. Coverslip with Permount

PROTOCOL 4:

Assessment of the Expression of RXRα, RXRβ, RXRγ in Breast Carcinomas by Immunocytochemistry

Antibodies: RXRα, RXRβ, RXRγ (Santa Cruz) Polyclonal
Kit: Vecta Elite ABC Kit (rabbit)
Slides from Formalin-fixed Paraffin Blocks

Prepare 1% BSA in PBS (0.05g BSA + 5 ml PBS)
Deparaffinize Slides: 3 x 5 min Xylenes
2 x 3 min 100% Ethanol
2 min 95% Ethanol
2 min 80% Ethanol
2 x 3 min dH_2O
10 min 3% H_2O_2
2 x 3 min H_2O
Antigen Retrieval: Microwave slides in 250 ml citrate buffer 3 min (until Boiling) at 100% powers followed by 12 min at defrost (245 watts). Cool slides in citrate buffer 15 min. Rinse slides 2 x 3 min dH_2O.
Blot Slides dry and circle tissue with PAP Pen (Let circles dry 2 min)
5 min PBS
Blocking: 20 min normal blocking sol. ABC kit (3 drops stock sol.[goat] to 10 ml PBS) *(yellow bottle)*
Staining:
Incubate slides with Primary Ab 1 hour at room temp.
Antibody dilutions: RXRα: 1:100 in 1% BSA in PBS
RXRβ: 1:100 in 1% BSA in PBS
RXRγ: 1:100 in 1% BSA in PBS
neg. control: nonimmune rabbit IgG
2 x 5 min PBS (Make goat-anti-rabbit and ABC at this time)
20 min Biotinylated Goat-antirabbit IgG (10 ml PBS + 25μl Goat-antirabbit, *blue bottle Vector Kit) Make sure real drop and not bubble!*
3x5 min PBS
30 min ABC (Vector Kit-10 ml PBS+2 drops A+2 drops B)
3x5 min PBS (Prepare DAB... 10 mg DAB/20 ml PBS, shake, filter, add 60 μl 3% H_2O_2)
2 min DAB
5 min dH_2O
Counterstain:
10 sec Gill's #1 hematoxylin
2 x 30 sec H_2O
20 sec Scott's tap H_2O substitute
5 min dH_2O
Mount:
1 min 70% EtOH, 1 min 95% EtOH, 2x1 min 100% EtOH
2x2 min Xylenes. Coverslip with Permount

PROTOCOL 5:

Assessment of DNA Aneuploidy and Cell Cycle Distribution in Mammary Tumors by Flow Cytometry

Processing the sample:

1. Disintegrate the tissue samples (fresh or frozen) ----Place a small piece of tissue (100-300 mg) in a petri dish and add 1.5 ml citrate buffer. Using a scalpel, mince the tissue into fine pieces. Using a pateur pipet further dissociate cells by flusing up and down several times.
2. Filter the suspension through 4 layers of gauze supported by a funnel into a 15 ml centifuge tube. Rinse petri dish and guaze with another 2 ml of citrate buffer. (Crush gauze with the pipet tip to save as many cells as possible.) Keep cold!! Place immediately in the refrigerator or on ice.
3. This suspension of cells in citrate buffer may be kept in a -80° C freezer for months.

Staining procedure: (Take solutions A,B & Citrate Buffer and bring to RT; thaw solution C, but keep cold on ice.)

4. Resuspend cells by lightly vortexing and spin down in a refrigerated centrifuge at 1000 rpm for 5 min.
5. Aspirate supernatant, leaving only about 300 μl above cell pellet.
6. **Resuspend the cells and take 100 μl and place in a fresh 12x75 culture tube. The remaining cells may be mixed with more citrate buffer and put back in the freezer for later use.)**
7. **Add 300 μl sol. A and mix gently. Incubate10 min at room temp. (make sure solns A & B are warmed to room temp.). Gently mix several more times over the 10 min.**
8. Put 250 μl sol. B and mix gently. Incubate 15 min at room temp. Again mix gently several times over the 15 min.
9. Put 250 μl sol. C and mix gently and place on ice. Incubate at least 30 min before taking measurements. (Measurements should be taken within 2 hrs after sol C has been added to the cell suspension.) Again keep cold!!!
10. Filter sample using 30 μm nylon mesh into fresh 12x75 culture tube.

Measure 10,000 cells per sample on flow cytometer.

Table 1: Retinoid Receptors in Breast Cancer (Immunocytochemistry - ICH)

Name/Histo #:	Age	Histology	Stage	Passage	ER	RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
1. MO/00-BC 1139	67	ICA		8	Neg	N+++	C/+	N/C+++	N/++	-	N/C+++
2. KS/00-BC 1122	42	ICA	Meta	5	Neg	N+/-	Neg	N/C +++	N/C ++	Neg	N ++
3. VD/00-BC 1121	52	ICA	Meta	5	Neg	N++	Neg	Neg	N ++	Neg	N ++
4.OP/00-BC 1123	48	ICA		4	Neg	N+++	N/C+/-	N +++	N +++	Neg	N/C +
5. BD/00-So10059	48	ICA		10	Neg	N+++	C ++	N +++	Neg	Neg	N ++
6. PS/00-BC 1124	46	ICA		2	Neg	N+++	N/C ++	N +++	N ++	N/E	N/C +++
7. DB/00-BC 1125	43	ICA		5	Pos	N++	N/C ++	N/C ++	N ++	N/E	N/C+/-
8. MC/00-BC 1127	58	ICA		4	Pos	N+++	N/C +	N/C +++	N +++	N/E	N/C ++
9. GJ/00-BC 1126	65	ICA	Meta	9	Neg	N++	Neg	N +++	N/C ++	N/E	N +/-
10.SG/00-BC 1134	41	ICA		1	Pos	N++	Neg	N/C +	N ++	Neg	N +/-
11.GS/00-BC 1130	61	ICA		2	Pos	N++	N/C +	N +++	N ++	N/E	N ++
12.BK/00- BC1131	53	ICA		1	Pos	N+++	N/C ++	N +++	N/C ++	N/E	N/C +++
13.SG/00-BC 1136	51	ICA	Man	3	Pos	N ++	C ++	N ++	N/C ++	N/C +	N +/-
14.TR/00-BC 1135	72	ICA		1	Pos	N++	C ++	N +++	N +++	N/C +	N ++
15.AA/99-BC 1137	45	ICA-1		6	Pos	N-	C ++	N +	N/C +	N/E	N/C+
16.WL/00BC 1138	50	ICA-2		8	Pos	N++	C +	N ++	N ++	N/C +	N/E
17.SK/00-BC 0113	44	Normal Breast		2	Pos	N+++	N ++	N +++	N +++	N +	N +++

Table 2

Summarized data on the expression of RARs and RXRs in breast tumors

Receptor	Number examined	Positive Nucleus	Positive Cytoplasm	Negative	
ER	16	7	-	9	
RARα	16	14		2	
RARβ	16	4	6	6	
RARγ	16	8	7	1	
RXRα	16	11	4	1	
RXRβ	11	1	5	5	
RXRγ	15	8	6	1	

Appendix II

Figure: 1, A, B, C, D, E, F, G, H

Figure: 2, A, B, C, D, E, F

Figures:

Fig. 1A: Breast carcinoma with nuclear localization of RARαa. Note that receptor is expressed in almost all tumor cells. The slide is counter-stained by hematoxylin, x 400

Fig. 1B: Expression of RARβ in a lobular carcinoma. Note the nuclear and cytoplasmic localization of the receptor. A high level of RARβ was also found in myoepithelial cells (arrow), x 200

Fig. 1C: Invasive ductal carinoma with RARβ expression in nucleus and cytoplasm. X 200

Fig. 1D: Ductal carcinoma with RARγ expression in both, nucleus and cytoplasm. There is variability in the nuclear staining. X 400

Fig. 1E: RARγ expression in a ductal carcinoma. Note the predominantly nuclear localization of the receptor, x 200

Fig. 1F: RXRα is expressed in normal ductal structures (left-hand side of the figure) and in tumor cells. In normal structures the cytoplasm is also lightly stained.

Fig. 1G: RXRβ expression in a parallel section from the same tumor. Note the nuclear and cytoplasma staining. X 200

Fig. 1H: RXRγ expression in a invasive ductal carcinoma. In most tumor cells the receptor is localized in the nucleus. X 200

Fig. 2A: RARα expression in breast cancer cells grown in vitro. There is a variability in the level of expression. In some cells the cytoplasm is also slightly stained x 40

Fig. 2B: RARβ is expressed in both, the cytoplasm and nucleus of tumor cells. Note significant variability in the shape of tumor cells.

Fig. 2C: RARγ was predominantly found in the nucleus of in vitro growing tumor cells. The cells were in the 6th passage of in vitro culturing.

Fig. 2D: RXRα was expressed both, in the nucleus and cytoplasm of tumor cells. There is variability in the form of cells as well as in the level of receptor expression.

Fig. 2E: RXRβ was mostly expressed in the nucleus of tumor cells. A light staining of cytoplasm close to the nucleus is also apparent.

Fig. 2F: RXRγ was found in the nucleus and cyytoplasm of tumor cells.

FIGURE 1

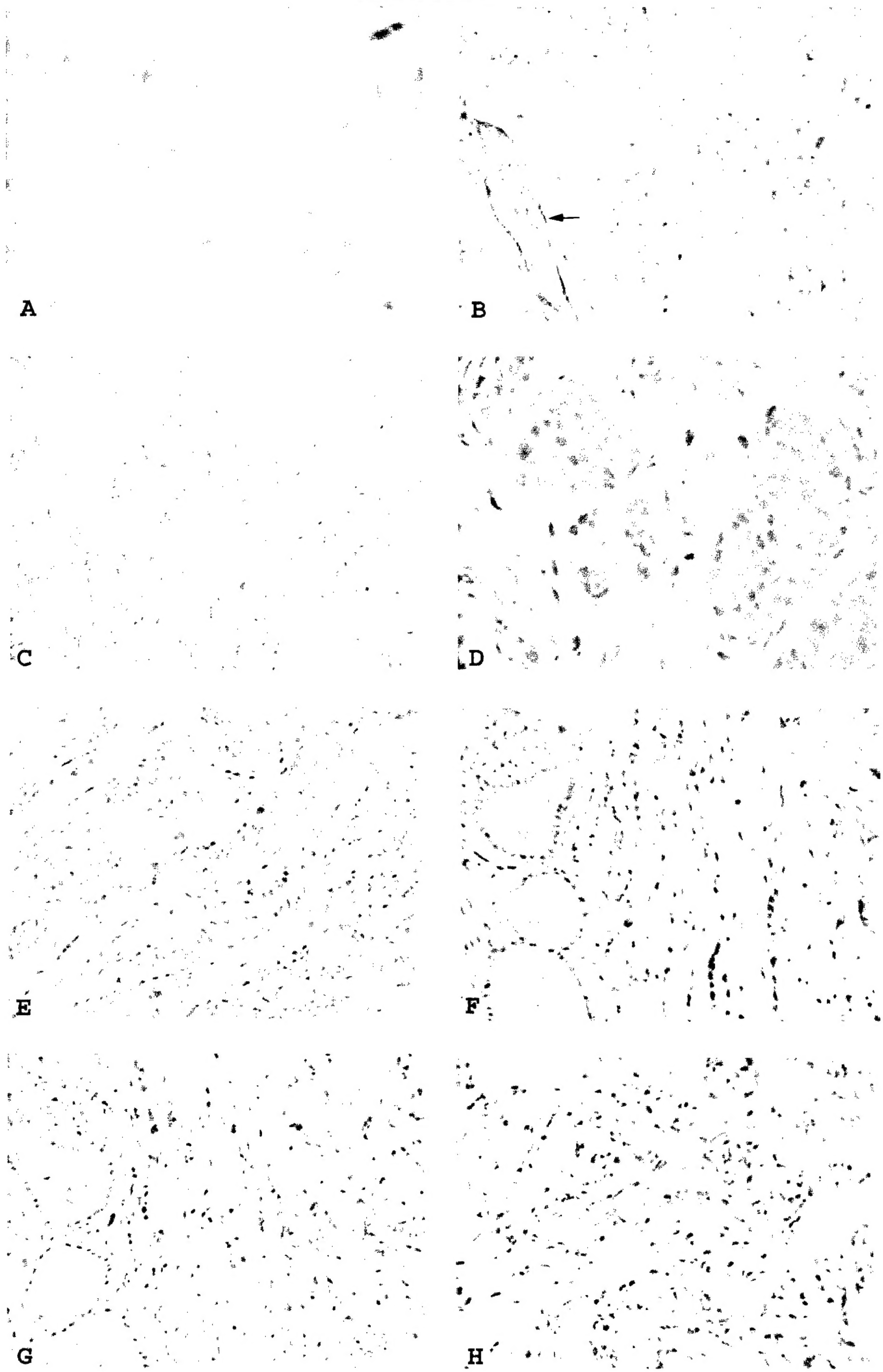

FIGURE 2